

FIG. 1

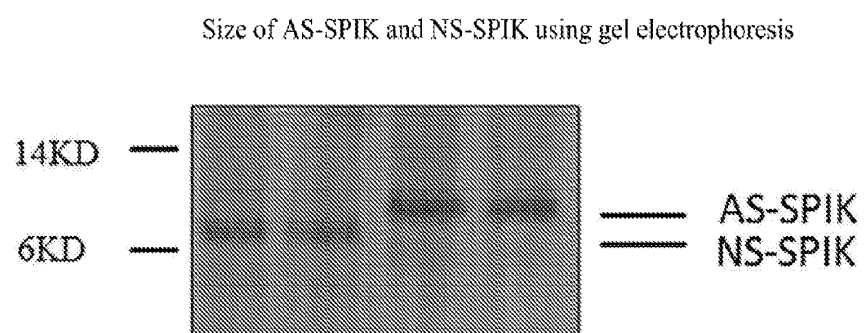


FIG. 2

Edman N-terminal analysis of AS-SPIK

Edman N-terminal analysis of AS-SPIK

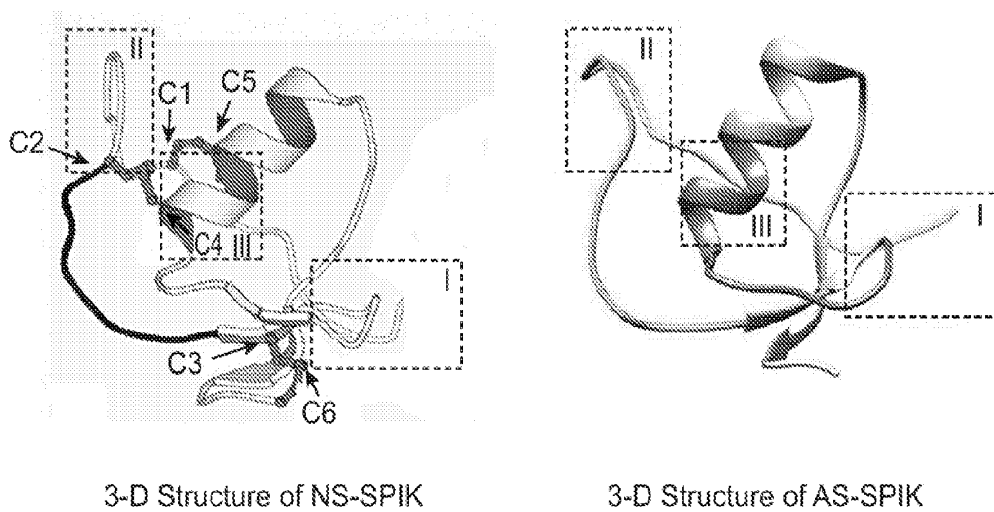
Cycle:	1	2	3	4	5
2 nd -6 th AA in N-terminal of native AS-SPIK :	K	V	T	G	I
N- terminal SEQ of AS-SPIK predicted by Edman:	(D/G)	V	T	G	(Q/T)

FIG. 3

AS-SPIK and NS-SPIK Amino Acid Sequence



The 3-D Structure of AS-SPIK and NS-SPIK



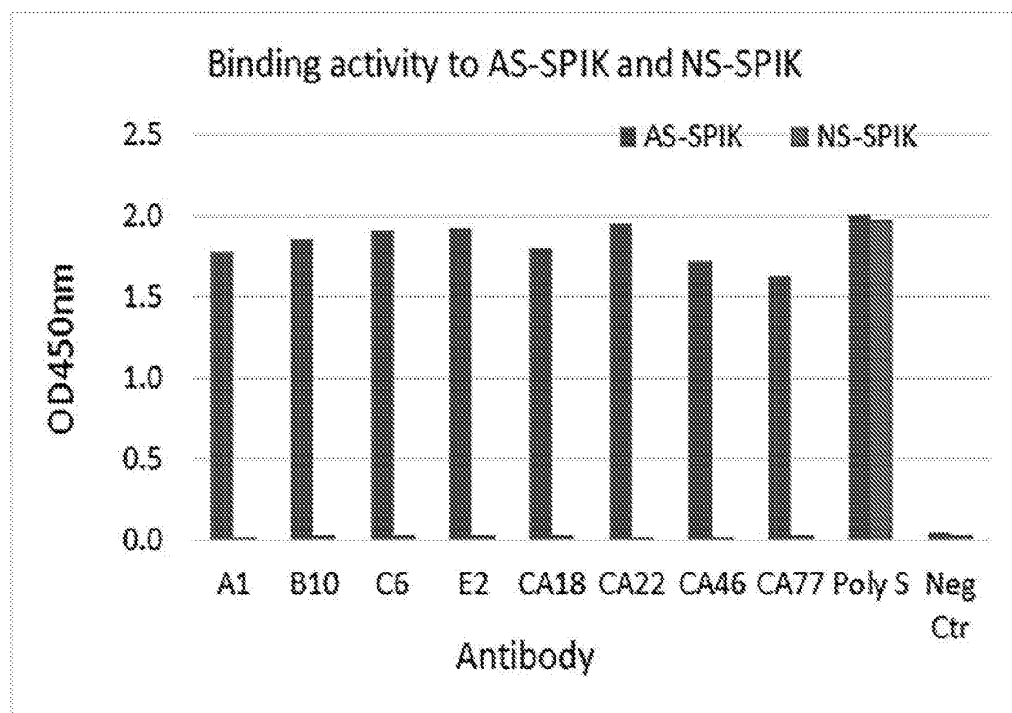
6 cysteines in SPIK which form 3 disulfide bond are pointed out.

FIG. 4

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FIG. 5

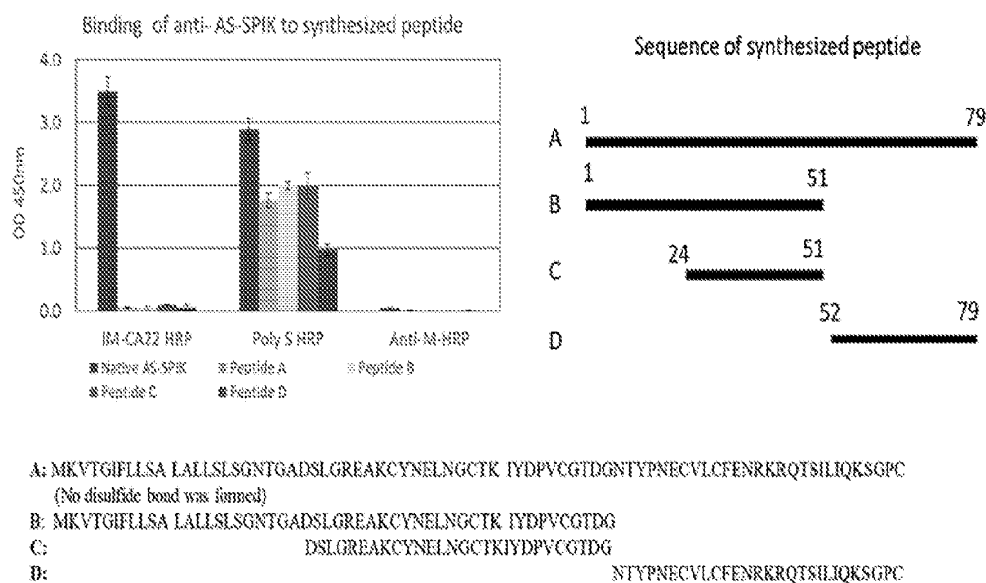
Monoclonal antibodies solely bind to AS-SPIK and not NS-SPIK



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FIG. 6

Binding of anti-AS-SPIK antibody to synthesized peptides



*Cysteines in peptide A was intended to be dysfunctional. No disulfide bond thus was formed.

The 3-D Structure (crystal mode) of epitopes of AS-SPIK

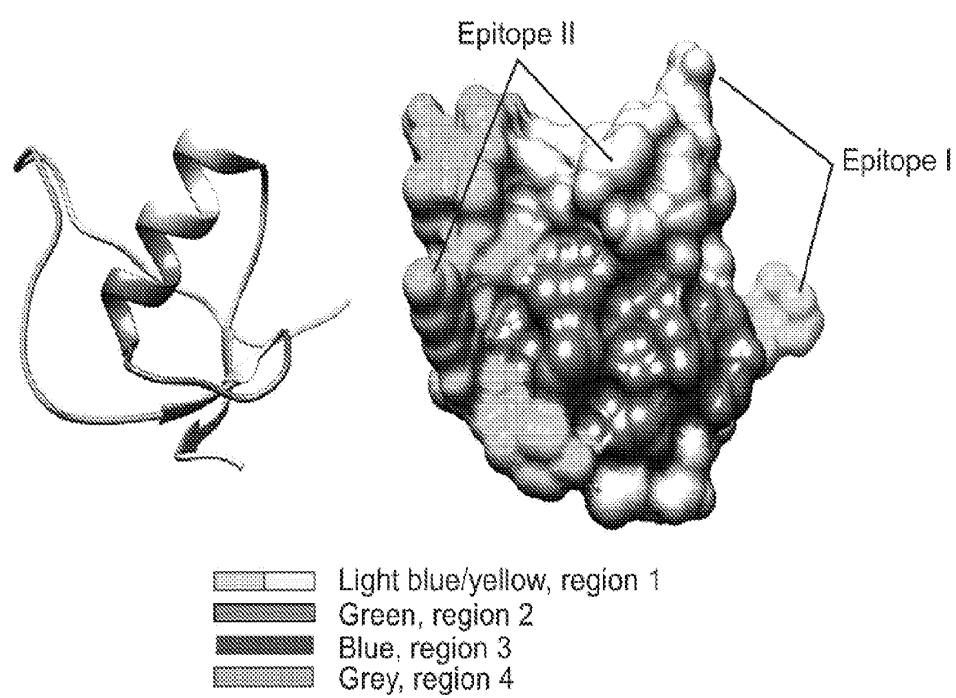


FIG. 7

FIG. 8

Inhibition test to locate the binding site of Class I anti-AS-SPIK antibodies

Name	Sequence	Inhibition %
LC-SPIK (10-30)	SALALLSLSGNTGADSLGREA	
SPIK4:	GNTGADSLG	6%
SPIK5:	SGNTGADSL	5%
SPIK6:	LSGNTGADS	6%
SPIK7:	SLSGNTGAD	6%
SPIK8:	LSLSGNTGA	11%
SPIK3:	LLSLSGNTGADSL	11%
SPIK2:	SLSGNTGADSLGR	10%
SPIK1:	SGNTGADSLGREA	9%
SPIK 9	ALLSLSGNTG	0%
SPIK 10	SALALLSLSG	-6%
SPIK 11	DLVPRGSPGI	-3%
SPIK 12	GLVPRGSPGI	-2%
SPIK 13	ZEDPGYRGRT	-4%

Peptides SPIK1-13 were synthesized. 0.5 µg/mL each peptide was added to prevent the binding of AS-SPIK with IM-CA22 (Class I antibody) in and ELISA test. Similar results were obtained using other Class I antibodies such as IMA1, IMB10 and IM-CA18, etc. The inhibition rate was determined by comparing OD value of with to without peptide. The sequence of each peptide is listed. SPIK 11-13 were peptides unrelated to LC-SPIK but have similar amino acids as SPIK3.

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FIG. 9

Conserved amino acids in the CDRs or Class I antibodies

CDRH1

SEQ ID NO: 19	A1:	SSNAI S
SEQ ID NO: 20	B10:	SSYGVS
SEQ ID NO: 21	CA22:	GYTFTDYYIN
SEQ ID NO: 22	CA18:	G FTFSRYAMS
Consensus:		S

CDRH2

SEQ ID NO: 23	A1:	AIG SSGS TYYASWAKS
SEQ ID NO: 24	B10:	S IWSGGTTDYASWAKS
SEQ ID NO: 25	CA22:	WIYPGSGNPIYNENFKD
SEQ ID NO: 26	CA18:	S I S IGGTYTYYPDSVKD
Consensus:		I GG Y K

CDRH3

SEQ ID NO: 27	A1:	RWENIGYTNVRLDL
SEQ ID NO: 28	B10:	RG-GYDYGYSNI
SEQ ID NO: 29	CA22:	EW-GCAMDS
SEQ ID NO: 30	CA18:	EDYGFYD
Consensus:		G Y

CDR of variable light ChainCDRL1

SEQ ID NO: 31	A1:	QASQSISTALA
SEQ ID NO: 32	B10:	QASES ISSYLS
SEQ ID NO: 33	CA22:	KSSQSLN-SGNQKNYLA
SEQ ID NO: 34	CA18:	KASQDVSTAVA
Consensus:		Q S

CDRL2

SEQ ID NO: 35	A1:	GASTLAS
SEQ ID NO: 36	B10:	RASTLAS
SEQ ID NO: 37	CA22:	GASTRES
SEQ ID NO: 38	CA18:	WASTRHT
Consensus:		AST S

CDRL3

SEQ ID NO: 39	A1:	QQGYSTSDVDNA
SEQ ID NO: 40	B10:	QQGYSVSNVDNI
SEQ ID NO: 41	CA22:	QS DYSHPYT
SEQ ID NO: 42	CA18:	HQHYSTYT
Consensus:		QQ YS

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FIG. 10

Conserved amino acids in the CDRs or Class II antibodies

CDRH1

SEQ ID NO: 43	C6:	S -YAI -S -W
SEQ ID NO: 44	E2:	SAYAI -S -W
SEQ ID NO: 45	CA46	G -YTFTSYWMQ
SEQ ID NO: 46	CA77	G -YTFSSNWIE
Consensus		Y S W

CDRH2

SEQ ID NO: 47	C6:	AINTYGGT -YYASWAKS
SEQ ID NO: 48	E2:	AINSG - GSAYYANWAKS
SEQ ID NO: 49	CA46	AIYPGDGDTRYTQKFED
SEQ ID NO: 50	CA77	QI FPGRDITNYNEKFKG
Consensus		AI G G Y

CDRH3

SEQ ID NO: 51	C6:	RDFDS-DAYTSASGGMDP
SEQ ID NO: 52	E2:	REDIY -DYGGAFDP
SEQ ID NO: 53	CA46:	GANYANIRFAY
SEQ ID NO: 54	CA77 :	RQEEFSDYYGSSHLYNYGMDY
Consensus:		R D

CDR of variable light ChainCDRL1

SEQ ID NO: 55	C6:	QASQSINNYLS
SEQ ID NO: 56	E2:	QASQGI SSYLS
SEQ ID NO: 57	CA46:	RASQDITNYLN
SEQ ID NO: 58	CA77:	RASQEI SGHLS
Consensus:		ASQ I YLS

CDRL2

SEQ ID NO: 59	C6:	RASTLAS
SEQ ID NO: 60	E2:	AATTLVS
SEQ ID NO: 61	CA46:	YTSRLHS
SEQ ID NO: 62	CA77:	AASILDS
Consensus:		AS L S

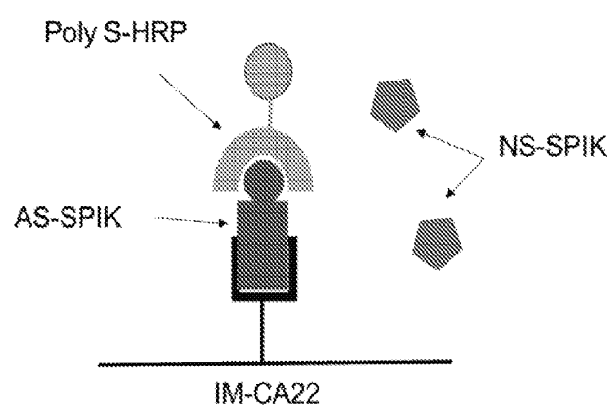
CDRL3

SEQ ID NO: 63	C6:	QQGYTSNVDNV
SEQ ID NO: 64	E2:	QQDYTTSNVDNT
SEQ ID NO: 65	CA46 :	QQGNTVPWT
SEQ ID NO: 66	CA77:	LQY - TDYPWT
Consensus:		QQ T

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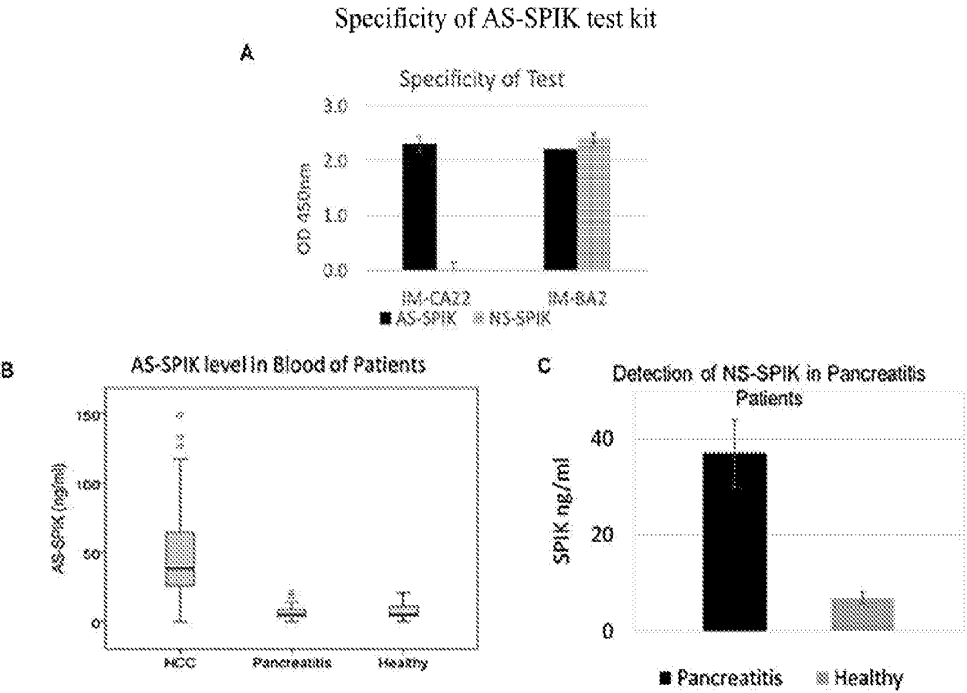
FIG. 11

Mechanism of ELISA test of AS-SPIK



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FIG. 12



Panel A. IM-CA22 binds specifically to AS-SPIK but not NS-SPIK, while antibody IM-BA2 binds to both AS-SPIK and NS-SPIK. **Panel B.** AS-SPIK is elevated in HCC, but not in pancreatitis or healthy patients. **Panel C.** Confirmation that NS-SPIK was elevated in the pancreatitis patients but did not interfere with the performance of the AS-SPIK-based detection kit.

FIG. 13

Table 1: The binding characteristics of Class I and Class II anti-AS-SPIK antibodies in a sandwich ELISA

A

Class II \ Class I	A1	B10	CA22	CA18	Poly S
C6	1ng/ml*	1ng/ml	1ng/ml	1ng/ml	10ng/ml
E2	1ng/ml	1ng/ml	1ng/ml	1ng/ml	10ng/ml
CA46	100ng/ml	100ng/ml	500ng/ml	500ng/ml	100ng/ml
CA77	500ng/ml	500ng/ml	500ng/ml	500ng/ml	100ng/ml
poly S	10ng/ml	10ng/ml	10ng/ml	10ng/ml	X

B

Class I	A1	B10	CA22	CA18	Poly S
A1	X	-	-	-	++++
B10	..**	X	-	-	++++
CA22	-	-	X	-	++++
CA18	-	-	-	X	++++

C

Class II	C6	E2	CA46	CA77	Poly S
C6	X	-	-	-	++++
E2	-	X	-	-	++++
CA46	-	-	X	-	++++
CA77	-	-	-	X	++++

A 96-well plate is coated with different antibody listed in the Column 1 at 0.1 µg/mL. The plate then reacts with AS-SPIK from S2-3 cells. After washing, the plate is incubated with HRP labelled signal antibodies as listed in Row 1, with varying concentrations.

* The concentration listed in each cell is the lowest concentration of signal antibody that gives a positive result (OD 450nm > 0.5).

**A “..” means that the signal antibody gave a negative result (OD450nm < 0.1) at the highest dose (500ng/mL).

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FIG. 14

Table 2: Potential anti-AS-SPIK binding regions as predicted by CLIPS study and the critical residues within these epitopes

Antibody Class I	Species	CLIPS predicted Region 1: S-29 AA	CLIPS predicted Region 4: 50-68
		GIFLLSALALLSLSGNTGADSLGREA	DGNTYPNECVLCFENRKQ
IM1 (IM-CA22)	Mouse	<u>SL</u> SGNTGADSLGRE	DGNTYPNECVLC
IM2 (IM-CA18/A9)	Mouse	<u>LL</u> SLSGNTGADSLGR	GNTYPNECVLCFENRKQ
IM7 (IM-A3)	Rabbit	GIFLLSALALL	CVLCFENRKQ
IM8 (IM-B10)	Rabbit	FLLSALALL	LCFENRKQ
Epitope I		FLLSALALLSLSGNTGADSLGREA	CVLCFENRKQ

Antibody Class II	Species	CLIPS predicted Region 2: 31-47	CLIPS predicted Region 4: 50-73
		KCYNELNGCTKIYDPVC	DGNTYPNECVLCFENRKQTSILQKSGPC
IM2 (IM-CA46/A3)	Mouse	KCYNELNGCTKIYD	GNTYPNECVL
IM4 (IM-CA77/6B)	Mouse	KCYNELNGCTKIY	NRKRQTSILQKSGP
IM6 (IM-C6)	Rabbit	NGCTKIYD	RKRQTSILQ
IM9 (IM-E2)	Rabbit	LNCTKIYDPV	NRKRQTSILQ
IM5 (Poly clonal AB, dominate binding region)	Sheep	CTKIYDPVCGT	CFENRKQTSILI
Epitope II		LNCTKIYD	NRKRQTSILQ

The amino acid(s) highlighted in the red text in Region 1 (Class I antibodies) are amino acids that were identified as critical for antibody binding via inhibition testing and epitope mapping. Any amino acids that are underlined lose >70% of their binding activity when replaced with a double Alanine mutation in Pepscan epitope mapping. Residues can fall into both categories (underlined and red).

FIG. 15

Table 3: Example Class I antibody sequences

Antibody A1	Amino Acid Sequence	SEQ ID NO:
A1 variable region heavy chain	LVAVLKGVQCQSVKESEGGLFKPTDTLTLTC TVSGFSLSSNAISWVRQAPGNLEWIGAIGSS GSTYYASWAKSRSTVTRNTNLNTVTLKMTSL TAADTATYFCARWENIGYTNVRLDLWGQGT LVTVSSGQPKAPSVFPLAPCCGDTTPSS	79
A1 variable region light chain	WLPGARCA YDMTQTPASVEVAVGGTVTIKCQ ASQSISTALAWYQQKPGQPPKLLIYGASTLASG VSSRFKSGSGTQFTLTISGVECADEAATYYCQQ GYSTSDVDNAFGGGTEG	80
A1 CDR H1	SSNAIS	19
A1 CDR H2	AIGSSGSTYYASWAKS	23
A1 CDR H3	RWENIGYTNVRLDL	27
A1 CDR L1	QASQSISTALA	31
A1 CDR L2	GASTLAS	35
A1 CDR L3	QQGYSTSDVDNA	39

Antibody B10	Amino Acid Sequence	SEQ ID NO:
B10 variable region heavy chain	LVAVLKGVQCQSVKESEGGLFKPTDTLT LTCTVSGFSLSSYGVS WVRQAPGKGLEW IGSIWSGGTTDYASWAKSRSTITRNTNEN TVTLKVTSLTAADTATYFCARGGYDYG ASNIWGPGLVTVSSGQPKAPS	81
B10 variable region light chain	LWLPGARCA YDMTQTPASVEVAVGGTVTIK CQASESISSYLSWYQQKPGQPPKLLIYRASTL ASGVPSRFSGSGSGTEFTLTISDGCDDAATY YCQQGYSVSNVDNIFGGGTEVVVKGDPVAPT VLIFPPSAD	82
B10 CDR H1	SSYGVS	20
B10 CDR H2	SIWSGGTTDYASWAKS	24
B10 CDR H3	RGGYDYGASNI	28
B10 CDR L1	QASESISSYLS	32
B10 CDR L2	RASTLAS	36
B10 CDR L3	QQGYSVSNVDNI	40

FIG. 15 (cont)

Table 3: Example Class I antibody sequences (cont.)

Antibody CA-22	Amino Acid Sequence	SEQ ID NO:
CA-22 variable region heavy chain	QIQLQQSGPELVKPGTSVKLSCKASGYTFDYYINWVK QRPQGQGLEWIGWIYPGSGNPIYNENFKDKATLTVDTSS TTAYLQLSSLTSEDSAVYFCAREWGCAMDSWGQGTSV TVSSAKTTAP SVYPLAP	75
CA-22 variable region light chain	DIVMTQSPSSLSVSTGEKVTMSCKSSQSLLNSGNQKNY LAWYQQKPGQSPKLLIYGASTRESGVPDRFTGSGSGTE FTLTISSVQAEDLAVYYCQSDYSHPYTFGGGTKLEIK	76
CA-22 CDR H1	GYTFDYYIN	21
CA-22 CDR H2	WIYPGSGNPIYNENFKD	25
CA-22 CDR H3	EWGCAMDS	29
CA-22 CDR L1	KSSQSLLNSGNQKNYLA	33
CA-22 CDR L2	GASTRES	37
CA-22 CDR L3	QSDYSHPYT	41

Antibody CA-18	Amino Acid Sequence	SEQ ID NO:
CA-18 variable region heavy chain	MNFVLSLIFLALILKGVQCEVQLVESGGGLVKPG RSLKLSCAASGFTFSRYAMSWVRQTPEKRLEGV ASISIGGTYYYPDSVKDRFTISRDNKNTLYLQ MNSLRSEDTAMYVCVREDYGFQDYWGQGLTVTVSS	77
CA-18 variable region light chain	DIVMTQSHKFMSTSVGDRVSITCKASQDVSTAVA WYQQKPGQSPKLLIYWASTRHTGVDPDRFTGSGSG TDYTLTISSVQAEDLALYYCHQHYSTYTFGGGTKLEIK	78
CA-18 CDR H1	GFTFSRYAMS	22
CA-18 CDR H2	SISIGGTYYYPDSVKD	26
CA-18 CDR H3	EDYGFQDY	30
CA-18 CDR L1	KASQDVSTAVA	34
CA-18 CDR L2	WASTRHT	38
CA-18 CDR L3	HQHYSTYT	42

FIG. 16

Table 4: Example Class II antibody sequences

Antibody CA-46	Amino Acid Sequence	SEQ ID NO:
CA-46 variable region heavy chain	QGHLLQSGAELARPGTSVKLSCKASGYTFTSYWMQWVK QRPQGQGLEWIGAIYPGDGDTRYTQKFEDKATLTADKSSS TAYMQLSNLASEDSAYYYCARGANYANIRFAYWGQGT LVTVSA	67
CA-46 variable region light chain	DIQMTQTTSSLSASLGDRVSISCRASQDITNYLNWYQQKP DGTVKLLIFYTSRLHSGVPSRFSGSGSGTNFSLTISNLEQE DIATYFCQQGNTVPWTFGGGKLEIK	68
CA-46 CDR H1	GYTFTSYWMQ	45
CA-46 CDR H2	AIYPGDGDTRYTQKFED	49
CA-46 CDR H3	GANYANIRFAY	53
CA-46 CDR L1	RASQDITNYLN	57
CA-46 CDR L2	YTSRLHS	61
CA-46 CDR L3	QQGNTVPWT	65

Antibody CA-77	Amino Acid Sequence	SEQ ID NO:
CA-77 variable region heavy chain	QVQLQQSGAELMKPGASVKISCKATGYTFSSNWIEWIK QRPGHGLEWIGQIFPGRDITNYNEKFKGKATFTADTSSN TAYMQLSSLTSEDSAVYYCARRQEEFSDYYGSSHLYNY GMDYWGQGTSVTVSS	69
CA-77 variable region light chain	DIQMTQSPSSLSASLGERVSLTCRASQEISGHLWLQQKP DGTIKRLIYAASILDSGVPKRFSRSGSDYSLTISNLESE DFADYYCLQYTDYPWTFGGGKVEIK	70
CA-77 CDR H1	GYTFSSNWIE	46
CA-77 CDR H2	QIFPGRDITNYNEKFKG	50
CA-77 CDR H3	RQEEFSDYYGSSHLYNYGMDY	54
CA-77 CDR L1	RASQEISGHLS	58
CA-77 CDR L2	AASILDS	62
CA-77 CDR L3	LQYTDYPWT	66

FIG. 16 (cont)

Table 4: Example Class II antibody sequences (cont.)

Antibody C6	Amino Acid Sequence	SEQ ID NO:
C6 variable region heavy chain	LVAVLKGVCQSVKESEGGLFKPTDALTLTCTVS GFSLSYAIWVRQAPGSGLEWIGAINTYGGTYYA SWAKSRSTITRNTNENTVTLKMTSLTAADTATYFC ARDFDSDAYTSASGGMDWGPGLVTVSSGQPKAP SFFPLAPCCGDTPR	71
C6 variable region light chain	WLPGARCAYDMTQTPASVEVAVGGTVTIKCQASQ SINNYLSWYQQIPGQPPKLLIYRASTLASGVSSRFKG SGSGTQFTLTISGVQCADAATYYCQQGYTSNVDNV FGGGTEVVVKGDPVAPTVLIFPPSAD	72
C6 CDR H1	SYAISW	43
C6 CDR H2	AINTYGGTYASWAKS	47
C6 CDR H3	RDFDSDAYTSASGGMDP	51
C6 CDR L1	QASQSINNYLS	55
C6 CDR L2	RASTLAS	59
C6 CDR L3	QQGYTSNVDNV	63

Antibody E2	Amino Acid Sequence	SEQ ID NO:
E2 variable region heavy chain	LVAVLKGVCQSVKESEGGLFKPTDLTLTCTV SGFSL SAYAISWVRQAPNGLEWIGAINSGGSA YYANWAKSRSTITRNTNLTNTVTLKMTSLTAAD TATYFCAREDIYDYGGAFDPWGPGLVTVSTGQ PKLHH	73
E2 variable region light chain	WLPGARCAYDMTQTPASVEVTVGGTVTIKCQAS QGISSYLSWYQQKPGQPPKLLIYAATTLVSGVSSR FKGSGSGTQFTLTISGVECADEAATYYCQQDYTT NVDNTFGGGTEVVVKGDPVAPTVLIFPPSAD	74
E2 CDR H1	SAYAISW	44
E2 CDR H2	AINSGGSAYYANWAKS	48
E2 CDR H3	REDIYDYGGAFDP	52
E2 CDR L1	QASQGISSYLS	56
E2 CDR L2	AATTLVS	60
E2 CDR L3	QQDYTTSNVDNT	64

FIG. 17

Table 5. The Serum AS-SPIK levels in clinical study with 512 samples

Cohort	Case No	Mean (ng/ml)	95% Confidence Interval for Mean	
			Lower Bound	Upper Bound
HCC	164	45.2	40.5	49.9
Early HCC	81	38.1	32.1	44.2
Cirrhosis	125	17.4	14.2	20.6
HBV/HCV	120	7.2	6.1	8.4
Pancreatitis	24	7.4	4.2	10.6
Healthy	79	7.4	6.2	8.6

FIG. 18

Table 6. Performance summary of AS-SPIK vs AFP in the detection of HCC

HCC vs liver disease	AUC	Sensitivity	Specificity	Cut-off (ng/ml)
AS-SPIK in HCC	0.87 (95%CI: 0.83 - 0.91)	80%	90%	21.5
AFP in HCC	0.70 (95%CI: 0.64 - 0.76)	52%	86%	20.0
AS-SPIK (Early HCC)	0.84 (95%CI: 0.79 - 0.89)	72%	90%	21.5
AFP in early HCC	0.61 (95%CI: 0.53 - 0.70)	42%	86%	20.0

FIG. 19

Table 7. AS-SPIK levels in early vs late stage HCC

Cohort	Sample Size	Mean (ng/ml)	95% Confidence Interval for Mean		P-value for comparisons between each group *
			Lower Bound	Upper Bound	
Early HCC	81	38.1	32.1	44.1	0.009
Later HCC	83	52.2	45.3	59.1	

* Differences are considered significant at $P < 0.05$

FIG. 20

Table 8. AS-SPIK levels in HCC, by BCLC stage

Cohort	Sample Size	Mean (ng/ml)	95% Confidence Interval for Mean		P-value for comparisons between each group *
			Lower Bound	Upper Bound	
Stage 0	6	33.7	17.1	50.2	0.250 – 0.998
Stage A	75	39.6	32.9	46.4	
Stage B	42	48.9	39.3	58.5	
Stage C	35	51.1	40.9	61.4	
Stage D	6	65.6	22.7	108.6	

* Differences are considered significant at $P < 0.05$